AD_____

Award Number: W81XWH-10-2-0003

TITLE: Vision Integrating Strategies in Ophthalmology and Neurochemistry (VISION)

PRINCIPAL INVESTIGATOR: Thomas Yorio

CONTRACTING ORGANIZATION: University of North Texas Health Science
Fort Worth, TX 76107

REPORT DATE: February 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-Feb-2012	Annual	28 Feb 2011 - 27 Jan 2012

4. TITLE AND SUBTITLE
Vision Integrating Strategies in Ophthalmology and Neurochemistry (VISION)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-2-0003

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Thomas Yorio

E-Mail: thomas.yorio@unthsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Texas Health Science
Fort Worth, TX 76107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall goal of the VISION project is to discover neuroprotective strategies in three separate mouse models of injury to the visual axis, in order to identify potential candidates for the treatment of combat eye injuries and preserve vision in our injured warfighters. We have established three different mouse models of ocular injury with different injury-initiating mechanisms (i.e. optic nerve crush, retinal ischemia/reperfusion, and chronic ocular hypertension). We have developed techniques to quantify damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus) that are damaged in these three models. We are testing neuroprotective agents and strategies, including neuroprotective estrogens, sigma-1 agonists, Brn3b, inhibitors of Jun N-terminal kinase (JNK), and inhibitors of protein stress to determine their efficacy in protecting the retina, optic nerve and superior colliculus from the damage induced by each of the 3 models.

15. SUBJECT TERMS
ocular injury; brain injury; neuroprotection; neuroprotective agents

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	26	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION:

This is the second year annual report for the VISION (Vision Integrating Strategies in Ophthalmology and Neurochemistry) project at UNTHSC. We currently have 6 PIs, 6 postdoctoral fellows, 5 graduate students, and 3 research technicians actively involved in this very ambitious project. In combat situations, traumatic eye injuries are frequent, leading to irreversible damage to the visual axis. The overall goal of the VISION project is to discover neuroprotective strategies in three separate mouse models of injury to the visual axis, in order to identify potential candidates for the treatment of combat eye injuries and preserve vision in our injured warfighters. We have established three different mouse models of ocular injury with different injury-initiating mechanisms (i.e. optic nerve crush, retinal ischemia/reperfusion, and chronic ocular hypertension). We have developed techniques to quantify damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus) that are damaged in these three models. We are testing neuroprotective agents and strategies, including neuroprotective estrogens, sigma-1 agonists, Brn3b, inhibitors of Jun N-terminal kinase (JNK), and inhibitors of protein stress to determine their efficacy in protecting the retina, optic nerve and superior colliculus from the damage induced by each of the 3 models. In addition, we are also evaluating time dependent, injury-induced changes in gene expression in the effected tissues to identify the major pathogenic pathways involved in order to develop new therapeutic approaches for neuroprotection and neuroregeneration. In the following report, we highlight the considerable progress made in this second year, including total neuroprotection by a JNK inhibitor in our model of retinal ischemia/reperfusion injury.

BODY:

Personnel: We currently have 12 PhD scientists, 3 research technicians, and 5 graduate students supporting this very ambitious project.

There are 6 PIs involved in this VISION research project. Dr. Abe Clark, the program Director for this project, is in charge of the CORE laboratory that runs the 3 mouse models of ocular injury and assesses structural and functional damage to the visual system (retina, optic nerve, and brain) by histology, SD-OCT and ERG. The CORE also runs gene expression profiling of the retina, optic nerve, and brain samples with disease progression in all 3 models of ocular injury. Dr. Clark is also testing the neuroprotective role of JNK inhibitors in these models. Dr. Marina Gorbatyuk has a proven record on using viral vector gene therapy to functionally and structurally rescue photoreceptor cells in rodent models of retinal degeneration. She brings expertise in gene therapy and in cellular stress due to protein misfolding. The unfolded protein stress response plays important roles in neurodegeneration pathogenesis, including in our models of ocular injury. We also will use viral gene delivery to retinal ganglion cells to validate newly discovered pathogenic pathways. Dr. Raghu Krishnamoorthy is testing the neuroprotective and neuroregenerative effects of over-expressing the transcription factor Brn3b in retinal ganglion cells in vitro and in vivo. Dr. James Simpkins is testing the neuroprotective effects of estrogen analogs in cultured cells and in our animal models of ocular injury. Dr. Tom Yorio is evaluating the potential neuroprotective effects of sigma-1 receptor antagonists. Dr. Robert Wordinger is helping with the histological assessments of retina and brain damage in all 3 animal models.

Six 6 postdoctoral fellows to support this project. Three of the postdocs (Zhang Zhang, PhD; Byung-Jin Kim, PhD; Yang Liu, PhD) are working in the CORE laboratory with PI Abe Clark and are responsible for establishing, characterizing and running the 3 experimental mouse models (see progress below) and quantitatively assessing damage to the retina, optic nerve, and superior colliculus in the brain. Everett Nixon, PhD is working with PI James Simpkins to screen and characterize neuroprotective estrogens in order to select the best candidates for in vivo testing. Dorota Stankowska, PhD is working with PI Raghu Krishnamoorthy to clone Brn3b into expression vectors and prepare AAV viruses to test the neuroprotective and neuroregenerative capablilies of Brn3b in our 3 animal models. Xiao Qin Wang, PhD is working with PI Marina Gorbatyuk to evaluate the neuroprotective roles of viral vectors targeting the protein stress pathway.

Three research technicians help with the animal models and with histological assessment of damage to the retina, optic nerve, and superior colliculus. Sandra Neubauer is a trained histology technician who has set up and runs our histology core facility. Holly Tebow and Terri Beckwith help run all 3 animal models as well

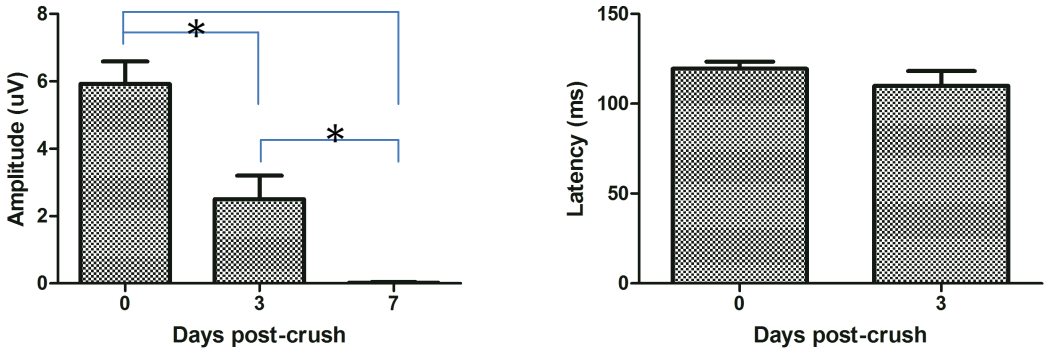
as quantitative assessment of damage to the retina, optic nerve, and superior colliculus.

We have 5 graduate (PhD) students working on the VISION project. Wanda Medina is working with Dr. Clark to study ocular and brain injury associated extracellular matrix remodeling (fibrosis and glial scar formation. Tasneem Putliwala is working with Dr. Clark to harvest RNA from the retina, optic nerve head, optic nerve, and superior colliculus at different time points in all 3 mouse models and will perform an extensive genomics study in order to identify pathogenic pathways and new potential therapeutic targets. She will collaborate closely with our U. Iowa bioinformatics colleagues for rigorous pathway analysis. Sean Silverman is working with Dr. Clark to evaluate the potential pathogenic role of the complement pathway in damage to the retina, optic nerve, and superior colliculus in all 3 mouse models. Nitasha Phatak is working with Dr. Krishnamoorthy to study the role of Brn3b in optic nerve regeneration in all 3 mouse models. Brett Mueller is a medical and PhD student working with Dr. Yorio to determine the neuroprotective effect of sigma-1 receptor agonists.

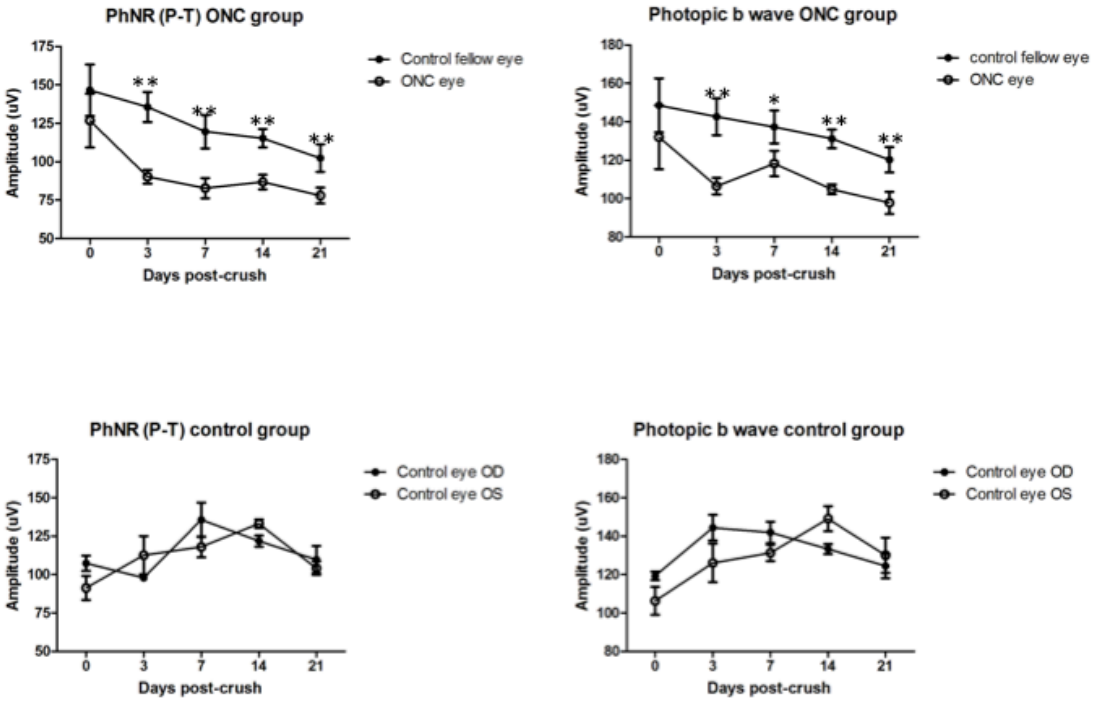
CORE Laboratory: Models of Ocular Injury

Mouse Model of Optic Nerve Crush (ONC): We have established the optic nerve crush (ONC) mouse model and investigated the time course of retinal ganglion cell (RGC) loss after optic nerve crush. Starting 7 days after intraorbital optic nerve crush, the loss of RGCs was continuous and cell number in the RGC layer was significantly lower in the ONC eyes. At 28 days post-crush, there were very few RGCs remaining in the retinas. Approximately 50% of the cells in the RGC layer are amacrine cells, so a 50% loss of cells in the RGC layer equates to an almost total loss of RGCs. We investigated the longitudinal effect of ONC injury in mice by measuring retinal thickness with spectral-domain optical coherence tomography (SD-OCT). The combined retinal thickness of nerve fiber layer (NFL) and inner plexiform later (INL) was measured. Nerve-crushed eyes showed statistically significant decrease ($p<0.01$) starting 7 days after optic nerve crush. To analyze the SD-OCT images more objectively and efficiently, we have set up the Beta site of MinerLite software for Mouse Segmentation and Thickness “Heat Map” reporting. The new software release will be available by the end of February. The previous data will be reanalyzed and confirmed with the new software. We have developed pattern electroretinography (PERG) techniques, which specifically measures RGC function, and determined the baseline characteristics as well as reproducibility of PERG as a tool to monitor progressive RGC dysfunction in ONC model. PERG responses were obtained from all recorded eyes of C57/BL6 mice. The PERG showed a prominent positive wave that peaked, on average, around 70 ms after each reversal of the visual pattern. However, only about 50% of recorded eyes of BALB/cJ mice had PERG responses, with smaller waveform ($p<0.05$) around 124 ms. Therefore, there are significant differences in PERG responses between mouse species, which was not previously recognized. PERGs were similar in overall waveform before and 3

days after crush. However, PERG amplitudes were significantly reduced 3 days after crush ($p<0.05$) and totally eliminated 7 days after crush. We also adopted the Flash ERG to follow the functional changes in RGCs after ONC using PhNR and pSTR responses. We have optimized the recording conditions and started a pilot study. The first pilot study will be done by the end of February. In addition to morphological and functional changes to the retina, there were significant effects in the brain region innervated by RGC axons. There was a progressive loss of neuronal volume assessed by black-gold staining of coronal sections through the superior colliculus starting at 2 weeks post crush. This clearly demonstrates that optic nerve axonal injury damages both the RGC soma and the target neurons in the brain, so **ONC is a model of both retinal and brain injury**.

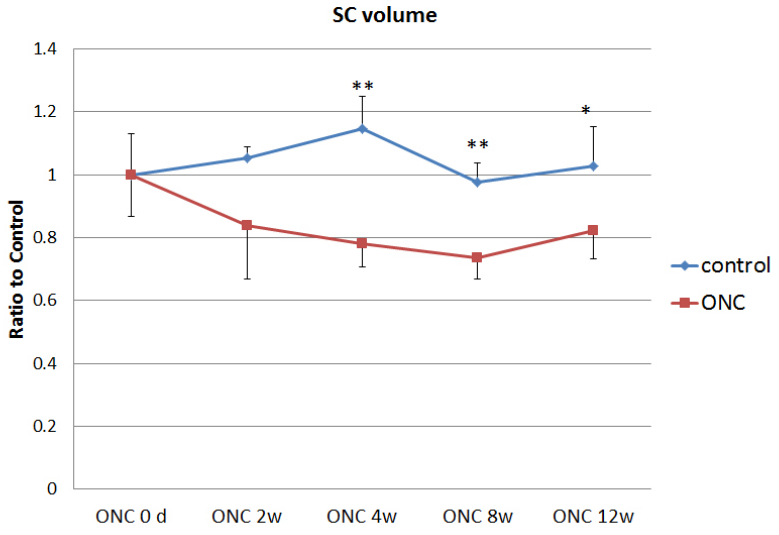


Progressive loss of RGC function shortly after ONC as assessed by PERG amplitude. The PERG amplitude was not measurable by 7 days post ONC. However, there was no change in PERG latency. * = $p<0.05$; *** = $p<0.001$



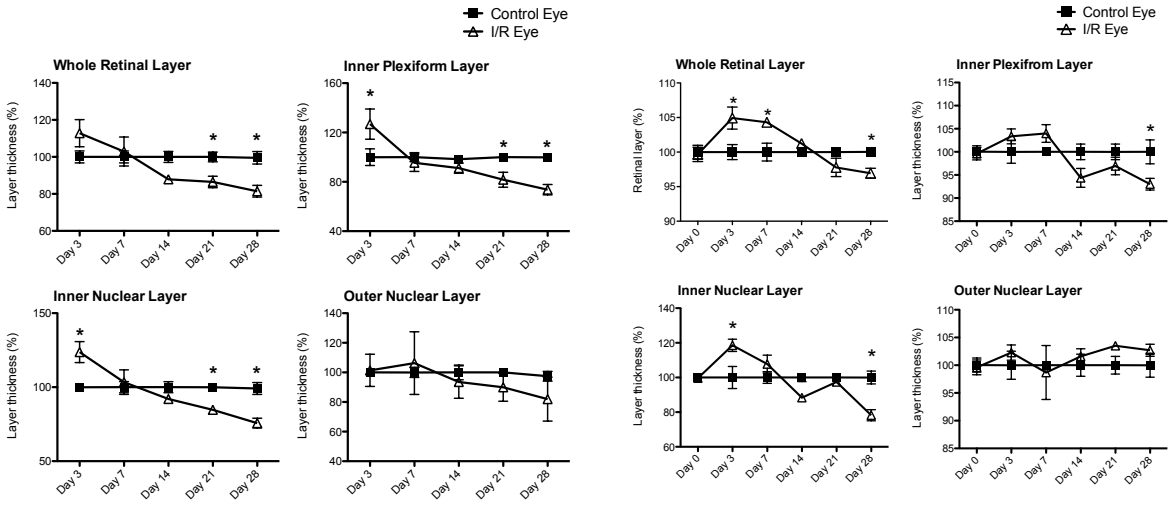
There was a progressive and significant decrease in the ERG photopic negative response (PhNR), measuring RGC function, after optic nerve crush. The was no change in the control eye. ** = p<0.01

ONC induced SC degeneration



ONC also damages the superior colliculus in the brain, which is the target of RGC axons. There was a significant loss in superior colliculus neural volume as assessed by black-gold staining on weeks 4-12. * = p<0.05, ** = p<0.01

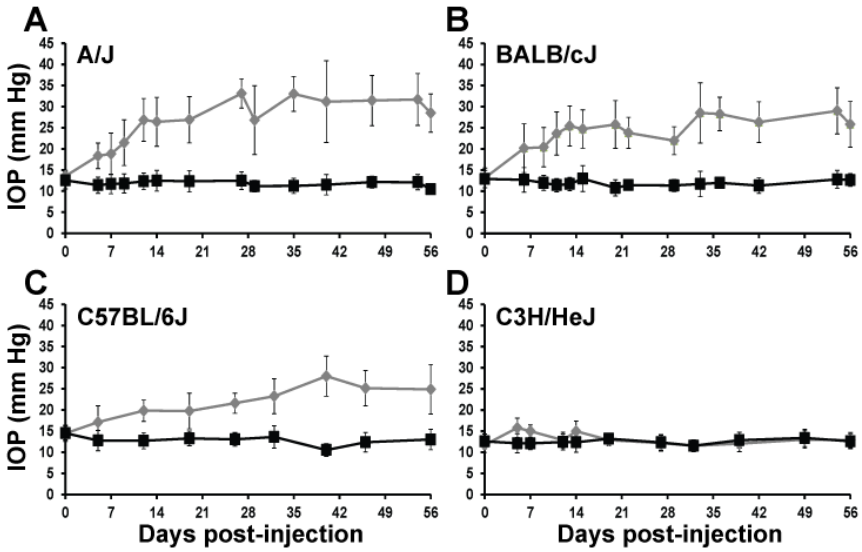
Mouse Model of Retinal Ischemia/Reperfusion (I/R) Injury: We established a mouse model of retinal ischemia/reperfusion (I/R) injury, and evaluated the progressive morphological and functional changes in the retina various time points (0, 3, 7, 14, 21, 28, and 35 days). Using SD-OCT scanning and H&E staining of retinal cross-sections, we found that whole layer thickness of retina was significantly increased 3 days after I/R injury (120 mmHg, 60 min), likely due to retinal edema, followed by a progressive significant decrease in retinal thickness through 28 days in I/R injured eye. We also found that these changes predominantly occurred in inner retinal layers (i.e. inner plexiform layer and inner nuclear layer). In addition, retinal detachment was observed at 3 and 7 day after I/R injury. Cell numbers in RGC layer also significantly decreased from day 14 after I/R injury. In scotopic ERG analysis, the amplitude and implicit time of b-wave was significantly impaired in I/R eye through 28 days in I/R injured eye. Interestingly, the a-wave in I/R eyes was significantly impaired through 21 days but recovered 28 and 35 days after injury, which may be correlated with recovery of retinal detachment after 14 day of I/R injury. Currently, we are preparing to characterize expression of molecular markers related with cell death, inflammation, and other biological processes in this model.



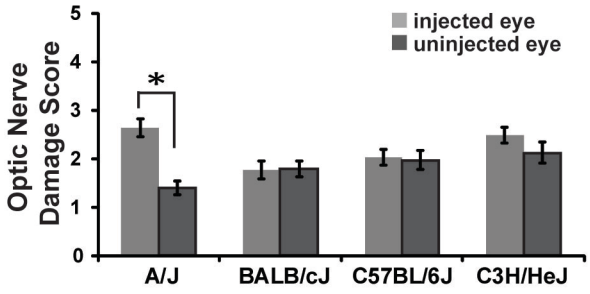
Analysis of changes in retinal layer thickness by histology (H&E staining) (left panel) and by SD-OCT analysis (right panel). There was increased retinal thickness 3-7 days post ONC, likely due to retinal edema, followed by a progressive decrease in thickness, especially in the inner retinal layers. * = $p<0.05$

Mouse Model of Chronic Ocular Hypertension: We developed a new mouse model of chronically elevated intraocular pressure (IOP) using a viral vector to over-express a human glaucoma transgene (mutant MYOC). Four mouse strains (A/J, BALB/cJ, C57BL/6J, and C3H/HeJ) were used in this study. Ad5.MYOC.Y437H (5 X 10^7 pfu) was injected intravitreally in one eye, with the uninjected contralateral eye serving as the control eye. Conscious IOP

measurements were taken using a TonoLab rebound tonometer. Optic nerve damage was determined by scoring PPD stained optic nerve cross sections. Retinal ganglion cell and superior colliculus damage was assessed by Nissl stain cell counts. Intravitreal administration of viral vector Ad5.MYOC.Y437H caused a prolonged, reproducible, and statistically significant IOP elevation in BALB/cJ, A/J, and C57BL/6J mice. IOPs increased from baselines of 12-15 mmHg to approximately 25 mmHg for 8 weeks ($p<0.0001$). In contrast, the C3H/HeJ mouse strain was resistant to Ad5.MYOC.Y437H induced IOP elevation for the 8-week time period. IOPs were stable (12-15 mm Hg) in the uninjected control eyes. We also determined whether there were any strain differences in pressure-induced optic nerve damage. Even though IOP was similarly elevated in three of the strains tested (BALB/cJ, C57BL/6J, and A/J) only the A/J strain had considerable and significant optic nerve damage at the end of 8 weeks, with an optic nerve damage score of 2.64 +/- 0.19 ($n=18$, $p<0.001$) in the injected eye. These results demonstrate strain dependent responses to Ad5.MYOC.Y437H-induced ocular hypertension and pressure-induced optic nerve damage. This novel mouse model provides an excellent resource to identify the initiating factors in RGC soma and optic nerve pathogenesis as well as the pathways involved in the disease progressing from the onset of elevated IOP to later stages of retinopathy and neuropathy. These data are currently submitted and are in review for publication. Future studies will include an extended time course study in A/J mice out to 6 months. Damage to the RGC's, optic nerve, and superior colliculus are currently being analyzed. A 24 week time course was also performed using this model system in A/J mice, and samples have been collected at 0, 4 weeks, 8 weeks, 12 weeks, 16 weeks, and 24 weeks post-injection. Retina, optic nerve head, and superior colliculus samples were processed for RNA isolation and microarray analysis. The optic nerves of each individual mouse were also collected to correlate pressure-induced damage with gene expression changes.



Transduction of mouse eyes with mutant glaucoma gene MYOC significantly elevated IOP in three mouse strains (A/J, BALB/cJ, and C57BL/6).

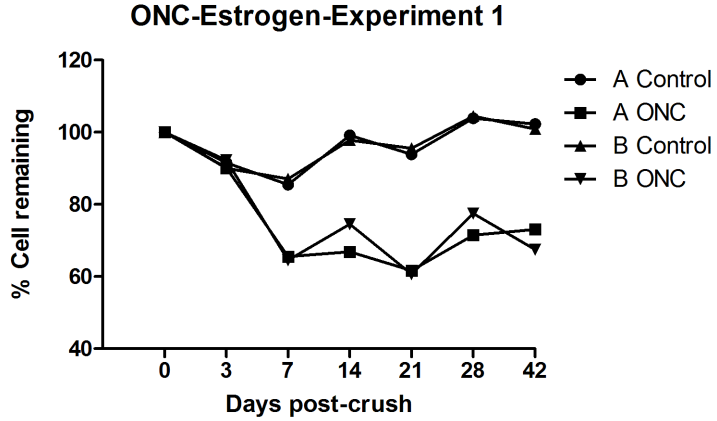


Differing mouse strain susceptibilities to pressure induced optic nerve damage. Sustained elevated IOP caused significant damage to the optic nerve in the A/J mouse strain. * = $p<0.05$

NEUROPROTECTION STUDIES

Neuroprotective Estrogens (Simpkins)

In Vivo ONC Model: Estrogen and estrogen analogs have been reported to have neuroprotective properties in the brain (Yi KD et al. Brain Res 2011;1379:61-70) and in cultured retinal neurons (Kumar DM et al. Free Radic Biol Med 38:1152-1163 and this report (see below)). A large single bolus of 17β-estradiol (group A) or vehicle (group B) was given to mice 2 hrs. prior to optic nerve crush injury, and potential neuroprotective effects were analyzed from 0-42 days post ONC. Cells in the retinal ganglion cell layer of Nissl stained retinal flat mounts were counted. Although ONC caused progressive loss of cells from the RGC layer, there was no significant differences in cell numbers between the vehicle and estrogen treated groups. Therefore, a single bolus of 17β-estradiol did not protect the retina from ONC injury. Additional studies are planned with more effective NP estrogen analogs and with daily dosing.



In Vitro Model: During the last 12 months, we have completed our in vitro assessment of a number of estrogen-related compounds for neuroprotective effects in 661W retinal neuronal cells and have made progress in establishing a primary retinal ganglion cell culture system in our laboratory. In this study, we tested the neuroprotective effects of 17β-estradiol (E2) and three synthetic

estrogen analogues (ZYC-26, ZYC-23, and ZYC-3) to examine their abilities to protect retinal neurons against glutamate toxicity. We first determine the presence of estrogen receptors (ER) in these 661W cells and observed by western blot analysis that 661 W cells expressed high level of ERα and modest levels of ERβ. We then optimized glutamate-induced cytotoxicity in 661W cells by determining the effects of increasing concentrations of glutamate. A dose-dependent loss of 661W cells was seen with an ED_{50} of about 4 mM. For assessment of the effects of estrogens on glutamate-induce cytotoxicity, we administered 5mM glutamate and the estrogen of interest. We observed that E2 was modestly cytoprotective, and then only at high concentrations (10μM). In contrast, estrogen analogs ZYC-26 and ZYC-3 were protective against a 5mM glutamate insult at concentrations as low as 100nM. The neuroprotective abilities of ZYC-26 and ZYC-3 were independent of ERα and ERβ as our previous studies had shown no binding to either ER and a lack of stimulation of estrogen responsive tissue in vivo. Also, in the present study, both ZYC-26 and ZYC-3 were ability to protect in the presence of ICI 182,780, a pan-ER antagonist with a high affinity for the estrogen receptor. As expected, ZYC-23, an inactive analogue of ZYC-26, was ineffective in protecting 661W cells at any concentration tested. Subsequently, we assessed two potent ER agonists, PPT (an ERα agonist) and DPN (an ERβ agonist). These compounds were ineffective at all concentrations tested in protecting 661W cells from glutamate toxicity. Finally, we tested the membrane ER receptor agonist G1 for neuroprotection in 661W cells. We observed a potent and efficacious activity of G1 in these cells. Interestingly, this protection was not antagonized by the membrane ER antagonist, G15. Likewise, the protective effects of E2, ZYC-26 and ZYC- 3 were not antagonized by G15 in 661W cells. These data suggest that the membrane ER does not mediate the effects of any of the estrogens tested.The relative potency of the compounds tested for protection of 661W cells was ZYC-26>G1>ZYC-3>E2>>>ZYC-23, PPT and DPN (Table 1). The most potent of these compounds, ZYC-3, ZYC-26 and G1 are non-feminizing compared to the inactive compounds; PPT, an ERα agonist, DPN, an ERβ agonist, and ZYC-23. The inverse relationship between potency in this 661W model and ER binding argues that neuroprotection and ER binding are clearly distinguishable processes. We have made similar observations concerning separation of ER binding and neuroprotection in hippocampal cell types, and in animal models of cerebral ischemia.

Table 1. Potency of estrogen-related compounds in protecting 661W cells rom glutamate-induced cell death.

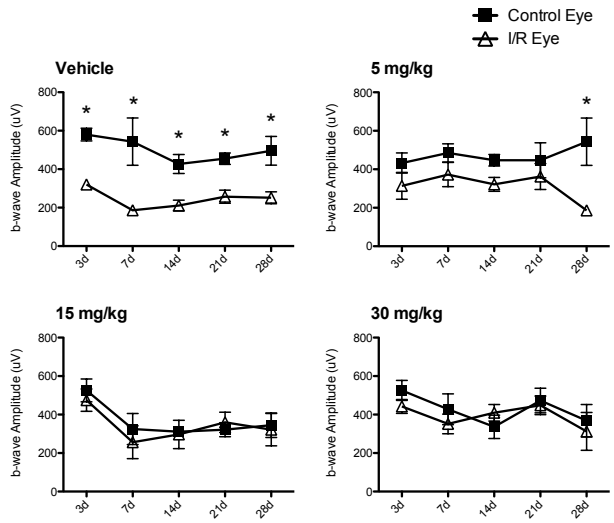
Compounds	ED50 (μM)
E2	4.4
ZYC-3	0.44

ZYC-26	0.065
G1	0.12
ZYC-23	>10
PPT	>10
DPN	>10

Future Directions: Primary rat retinal ganglion cells (RGC) from p4 rat pups will be isolated and cultured in vitro for 7 days. These cells will be used for glutamate toxicity assay since they are post synaptic neurons that express glutamate receptors, and they should therefore be susceptible to glutamate toxicity. A concentration ranging from 1 to 10mM of glutamate will be used to determine the best concentration for further experimentation. Once we are able to induce cell damage, cells will be pretreated with estradiol (E2) and its active analogs, ZYC-2 6 and ZYC-3 to determine whether they can ameliorate cell viability in the face of glutamate toxicity. We also intend to investigate the signaling pathways involved in cellular damage as well as the pathways involved in providing neuroprotection to these RGCs. These agents will also be tested in our in vivo models of ocular injury.

JNK Inhibitor Neuroprotection (Clark)

The Jun N-terminal kinase (JNK) pathway is activated and plays a crucial role in neuronal damage, including ischemia-related neuronal pathogenesis. Therefore, we examined the effect of the selective JNK inhibitor SP600125 on our mouse model of retinal ischemia/reperfusion (I/R) injury. Four groups of mice (n=5 mice per group) were given daily intra-peritoneal injections of three different doses of SP600125 (0, 5, 15, 30 mg/kg), while the negative control group received the formulation vehicle. Unilateral retinal I/R injury (intraocular pressure elevation of 120 mmHg for 60 minutes) was given in the left eye of each mouse. Retinal function was monitored using scotopic ERG at 0, 3, 7, 14, 21 and 28 days after I/R injury. I/R injury caused early and significant decreases in ERG amplitude and increases in implicit time in the vehicle treated group. However, SP600125 dose-dependently protected the retina from I/R injury, with the highest 2 doses providing total protection of ERG a- and b-wave function out to the end of the study (28 days). At the end of this study, retina samples were collected from each mouse, and we are currently evaluating the effects of this JNK inhibitor on I/R-induced morphological changes in retinal layer thickness and number of cells in the RGC layer. We will determine whether SP600125 also morphologically protected the retina from I/R injury. Very few agents have provided this level of protection in this severe acute model of retinal injury.



JNK inhibitor SP600125 dose dependently protected retinal ERG b-wave amplitudes in the mouse retinal ischemia/ reperfusion. Retinal I/R significantly decreased b-wave amplitudes on days 3-28 post injury in the vehicle group. The 2 highest doses of SP600125 (15 and 30 mg/kg) totally protected the ERG b-wave responses at all time points. * = $p<0.05$

Brn3b Neuroprotection/Neuroregeneration (Krishnamoorthy)

A recombinant adeno-associated viral (AAV) vector encoding the POU domain transcription factor, Brn3b (POU4F2), was produced in the laboratory using a commercially available kit (Stratagene, La Jolla, CA). Recombinant AAV viral particles encoding Brn3b were further purified by column chromatography using a commercially available kit (ViraBind AAV Purification Kit, Cell Biolabs Inc., San Diego, CA). Preliminary studies were carried out to test the ability of the AAV-Brn3b viral construct to promote optic nerve regeneration *in vivo* in the Morrison rat model of ocular hypertension. Briefly, adult male retired breeder Brown Norway rats were intravitreally injected in the left eye with approximately 1×10^9 viral particles encoding either the empty vector (AAV-MCS) or the transcription factor, Brn3b (AAV-Brn3b). One week following administration of the viral vectors, intraocular pressure was elevated in the left eye by infusion of approximately 50 µl of 1.8 M NaCl through the episcleral veins to sclerose the aqueous outflow pathway. After elevation of intraocular pressure, the rats were maintained for 3 weeks and then sacrificed. Rat eyes were enucleated, fixed in 4% paraformaldehyde and five micron thick sagittal retina sections through the optic nerve head were obtained. Immunohistochemical analysis of Brn3b expression was carried out by staining with a custom-made Brn3b antibody (Antibody Research Corporation, St Charles, MO) and visualized using appropriate Alexa conjugated secondary antibodies. It was found that administration of AAV vector encoding Brn3b produced an appreciable increase in Brn3b expression in RGC layer of rat retinas, compared to rats that were administered the empty vector viral construct.

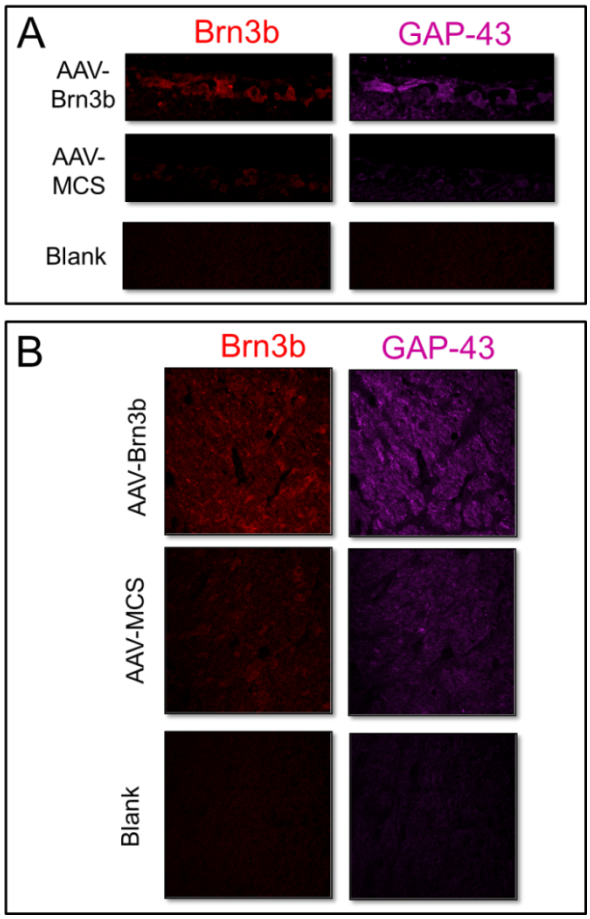


Figure 1. Immunohistochemical detection of Brn3b and GAP43 expression in the retina and optic nerve head of rats injected intravitreally with either an adeno-associated viral vector encoding transcription factor Brn3b (AAV-Brn3b) or an empty vector (AAV-MCS) and IOP elevated for 3 weeks.
A). *Left panel* depicts virally mediated increase in Brn3b expression observed in RGC layer of retina sections by immunostaining using a custom-made Brn3b antibody, compared to those in rats administered an empty vector. *Right panel* shows GAP43 expression analyzed by immunohistochemical staining which was increased in AAV-Brn3b treated rats, compared to AAV-MCS (control vector) injected rats.
B). Left panel shows Brn3b staining in optic nerve head of rats injected either with AAV-Brn3b or AAV-MCS (empty vector). Right panel shows GAP43 staining in optic nerve heads of rats injected with either AAV-Brn3b or AAV-MCS. Control sections (Blank) which were immunostained with the secondary antibody alone (excluding the primary antibody) showed minimal staining.

Other retinal sections from the same set of rats were stained for GAP43, a protein, which is concentrated in growth cones of developing and regenerating axons. As seen in **Figure 1A**, an increased staining for GAP43 was observed in retinal ganglion cells of rats intravitreally injected with the Brn3b expression vector, compared to that seen in rats injected with the control vector. Interestingly, increased staining for both Brn3b and GAP 43 was also observed in the optic nerve head at the transition zone posterior to the lamina cribrosa in rats injected with the AAV-Brn3b vector **(Figure 1B)**. These observations suggest the possibility of neuroregenerative effects mediated by transcription factor Brn3b following damage to the axons at the lamina cribrosa. Various markers of neuroregeneration were also tested to determine if upregulation of Brn3b in retinal ganglion cells could elicit increased expression of these proteins including Growth-associated protein 43 (GAP43), acetylated tubulin α1 (Tuba 1), and actin binding LIM protein (abLIM). As seen in **Figure 2A**, administration of the AAV-Brn3b viral vector increased the expression of Ab-LIM (a cytoskeleton interacting protein which is implicated in axon elongation through its effects on lamellipodia and filopodia formation). Overexpression of Brn3b also increased acetylated tubulin α1 (Tuba 1) expression mainly in the nerve fiber layer of the retina in rats administered the AAV-Brn3b vector. Similarly, increased immunostaining for the neuroregeneration markers, Ab-LIM and Tuba 1 was observed in the optic nerve

head at the transition zone in rats administered the AAV-Brn3b vector **(Figure 2B)**. These observations have implications for the ability of transcription factor Brn3b to promote neuroregeneration following optic nerve axonal injury in rats. Further experiments will be carried out to determine the ability of transcription factor Brn3b to promote axonal regeneration following optic nerve damage in three rodents models of injury to the visual axis.

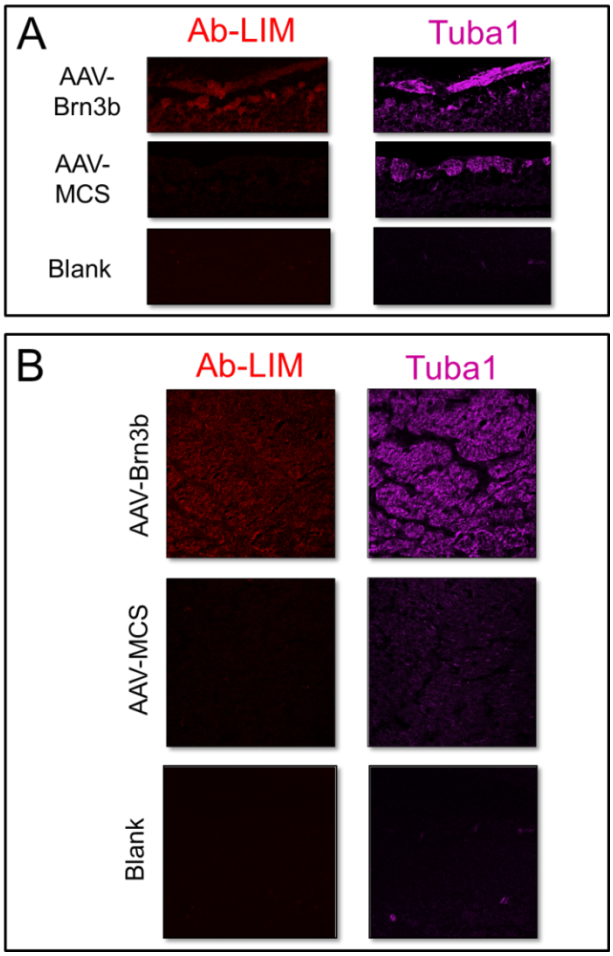
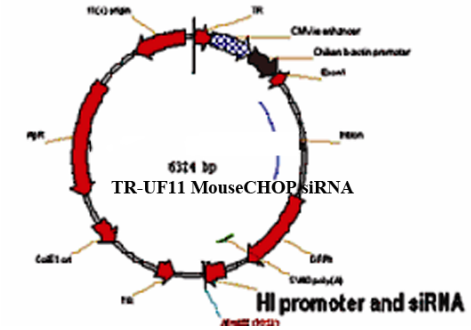


Figure 2. Immunohistochemical detection of Brn3b and GAP43 expression in the retina and optic nerve head of rats injected intravitreally with an adeno-associated viral vector encoding transcription factor Brn3b (AAV-Brn3b) or an empty vector (AAV-MCS) and IOP elevated for 3 weeks.
A). Left Panel depicts immunostaining for abLIM in RGC layer of rats administered an overexpression vector for Brn3b (AAV-Brn3b) or an empty vector (AAV-MCS). Right panel shows Tuba 1 expression analyzed by immunohistochemical staining in rat retinas. Tuba 1 immunostaining was increased in retinas of AAV-Brn3b injected rats, compared to that seen in empty vector (AAV-MCS) injected rats. **B)**. *Left Panel:* Optic nerve heads stained for Ab-Lim in AAV-Brn3b and AAV-MCS injected rat eyes. *Right Panel:* Tuba 1 staining in optic nerve heads of AAV-Brn3b and AAV-MCS injected rats. Control sections (Blank) which were immunostained with the secondary antibody alone (excluding the primary antibody) showed minimal staining.

Neuroprotection with AAV.BiP and AAV.CHOPshRNA (Gorbatyuk)

Many neuronal diseases are associated with insufficient protection of misfolded protein processing by the endoplasmic reticulum associated degradation (ERAD) pathway, which leads to activation of the unfolded protein response (UPR). If UPR does not sufficiently handle this protein stress, the cell proceeds to apoptotic death. In addition to the role of ERAD/UPR in retinal degeneration (RD) models, a recent report has shown that ERAD/UPR is activated in the ONC model and that suppression of UPR is neuroprotective (Hu Y et al. Neuron 2012;73:445-452). We are generating expression vectors for the molecular

chaperone BiP, which has been shown to protect the retina in RD models. The full-length cDNA of the human BiP/GRP78 (NM_005347) was inserted in an AAV plasmid with serotype 2 terminal repeats. AAV2 has tropism for retinal ganglion cells, which are the targets for neuroprotection in all of our 3 mouse models of ocular injury. The Bip/Grp78 expression was controlled by the chicken β-actin promoter and CMV immediate early enhancer (the CBA promoter). BiP mRNA was processed from the long transcript by splicing of the intron following the first β-actin exon, which does not code for protein. The maturation and stabilization of BIP mRNAs were supported by the SV40 polyA signal in the construct. The AAV vector was packaged in serotype 2 that is known to have efficient transduction of the ganglion cell layer following intravitreal injection.



Design of siRNA expression cassette. Sequence encoding siRNa targeting mouse CHOP protein under control of human H1 promoter was inserted in AAV plasmid.

Another therapeutic target for the ERAD/UPR mediated protein stress is knockdown of the proapoptotic factor CHOP. Based on results with siRNAs targeting mouse/rat Chop mRNAs, we designed an AAV vector expressing the most effective target specific siRNA sequences silencing the mouse Chop gene. The above figure shows the map of AAV-Chop siRNA. Currently a large-scale preparation of this vector is in a production. Plasmid DNA will be packaged in AAV with serotype 2.

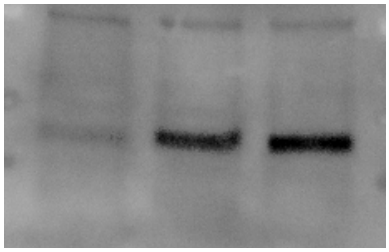
Neuroprotection by Sigma-1 Receptor (Yorio)

The progress over the last year has been focused on identifying important molecular targets and pathways that are important for the survivability of retinal ganglion cells (RGCs). Ischemic insult to primary RGCs induces changes in proteins that are essential for neuronal cell survivability and synaptic plasticity. These proteins include pCREB, pERK1/2, pDRP1, and pBAD. This project has been geared at trying to identify molecular targets that can help ameliorate the effects of ischemia, and help to protect RGCs through the induction of pCREB, pERK1/2, pDRP1 and pBAD. This study has led to the identification of how important NMDA stimulation is for the survival of RGCs. Doses that have shown to be excitotoxic in primary hippocampal neurons through the decrease in

pCREB have actually shown to be increased by NMDA in primary RGCs (Figure 1). Additionally, the blockade of NMDA receptors by MK801 completely abolishes the endogenous expression and NMDA induced expression of pCREB (Figure 2). These findings go against the NMDA excitotoxic hypothesis that has long been the driving force behind many neuroprotective therapeutic targets for the treatment of Glaucoma. Further experiments are needed to be performed, but it appears that RGCs are not only invulnerable to NMDA excitotoxicity, but heavily depend upon this signaling for their survivability. This may help explain why NMDA antagonist have not been protective in human studies to date. This data also goes against what has been shown in primary cortical neuronal cultures. Therefore, because RGCs appear to be able to handle "excitotoxic doses" of NMDA, the next question that needs to be addressed is why RGCs possess this special ability, and what makes them different from cortical excitatory neurons. And do other receptors on RGCs cause excitotoxicity through calcium overload? The molecular characterization of NMDA receptors and non-NMDA receptors can help to identify potential molecular targets that can be used to protect RGCs from diseases like Glaucoma.

Additionally, this research has also led to the identification of pentazocine, a sigma-1 receptor agonist, which has shown to be neuroprotective in RGCs from ischemic insult. It appears that this agonist induces the induction of pCREB in primary RGCs (Figure 3), and may be one of the neuroprotective mechanisms employed by this agonist to protect RGCs from pathological stressors.

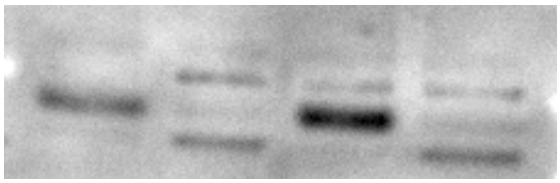
NMDA Induced Expression of pCREB



Control No Treatment	+	-	-
6 Hour treatment of NMDA 100μM	-	+	-
6 Hour treatment of NMDA 1mM	-	-	+

Figure 1: Excitotoxic doses of NMDA induced pCREB expression assessed by western immunoblotting in primary RGCs after 6 hours of treatment.

Antagonizing NMDA Receptor Induces Changes Expression of pCREB



Control (No NMDA Treatment)	+	-	-	-
MK801 Treatment (1μM)	-	+	-	+
6 Hour treatment of NMDA 100μM	-	-	+	+

Figure 2: MK801 treatment for 6 hours on primary RGCs abolished endogenous levels of pCREB and blocked NMDA induced expression of pCREB.

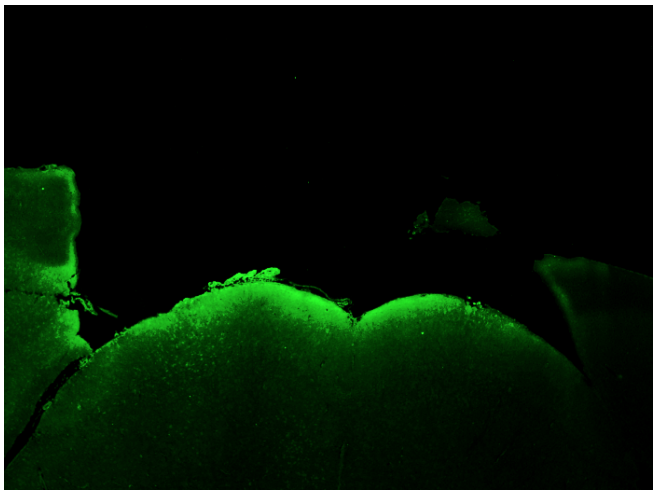
Pentazocine Induction of pCREB in Primary RGCs

Control	+	-
6 hr Pentazocine Treatment (1μM)	-	+

Figure 3: 6 hours of treatment of pentazocine induced the expression of pCREB.

Complement as a Neuroprotective Target (Clark)

Activation of the complement system occurs in several types of neuronal damage, including damage to retina (Howell G et al. J Clin Invest 2011;121:1429-1444). Complement activation, especially C1q, is thought to be one of the earliest pathogenic pathways activated, where it is involved in remodeling neuronal synapses. Depletion of C1q was neuroprotected the retina and optic nerve from damage in a mouse model of glaucoma (Howell et al. 2011). We have been investigating the role of C1q and C3 in neuronal damage to the superior colliculus and have shown increased expression of C1q in the superior colliculus of mice with chronically elevated IOP (see below). We will use C1q and C3 knockout mice to determine whether depletion of these complement factors is neuroprotective in our 3 mouse models of injury to the visual axis.



C1q immunostaining of the outer superior colliculus, the neuronal target in the brain for retinal ganglion cell axons. Immunostaining is more intense in the hemisphere that is innervated by the eye with chronic ocular hypertension.

Identification of Molecular Pathogenic Pathways and New Neuroprotective Strategies

Changes in Retinal Gene Expression in Optic Nerve Crush Model: RNA was extracted from retina, optic nerve, and superior colliculus (visual center in the brain) samples from eyes and brains of mice subjected to ONC injury at 0. 3. 7, 14, 21, and 28 days post ONC injury. RNA quality was checked using Agilent bioanalyzer chips and mRNA expression was determined using the Affymetrix mouse gene array platform at the DNA CORE Laboratory at U. Iowa. Data were analyzed using Partek and DAVID software packages. There were temporal changes in retinal gene expression as grouped by the following DAVID categories. Differential expression of select target genes has been confirmed by qPCR and several new potential neuroprotective strategies (e.g. therapeutic increased neuritin expression) will be tested in this model.

GENE ONTOLOGY	CLUSTERS	P VALUE	ENRICHMENT SCORE
MOLECULAR FUNCTION	STRUCTURAL EYE PROTEIN	1.90E-06	3.33
	EYE DEVELOPMENT	4.50E-03	2.84
	EXTRACELLULAR MATRIX BINDING	5.30E-03	1.95
	CALCIUM ION BINDING	3.30E-02	0.64
	STRUCTURAL EYE LENS PROTEIN	2.20E-11	7.07
	STRUCTURAL MOLECULAR ACTIVITY	4.60E-06	7.07
BIOLOGICAL PROCESS	RESPONSE TO WOUNDING	1.00E-04	2.37
	INFLAMMATORY RESPONSE	3.80E-04	2.37
	DEFENSE RESPONSE	5.90E-04	2.37
	POSITIVE REGULATION OF IMMUNE SYSTEM RE	1.20E-02	1.52
	RHO PROTEIN SIGNAL TRANSDUCTION	5.90E-03	1.11
	REGULATION OF SIGNAL PROLIFERATON	2.70E-02	1.03
	DEFENSE RESPONSE	5.40E-04	1.79
	INFLAMMATORY RESPONSE	1.60E-02	1.79
	RESPONSE TO WOUNDING	4.70E-02	1.79
	SENSORY PERCEPTION	8.80E-03	1.43
	NEUROLOGICAL SYSTEM PROCESS	2.30E-02	1.43
	G-PROTEIN COUPLED RECEPTOR SIGNALING PAT	3.90E-02	1.43
	MACROMOLECULAR COMPLEX ASSEMBLY	1.30E-02	1.27
	DNA PACKAGING	3.10E-02	1.27
	POSITIVE REGULATION OF PROTEIN KINASE ACT	4.50E-02	0.92
CELLULAR COMPONENT	CELL JUNCTION	2.00E-02	1.3
	EXTRACELLULAR REGION PART	3.20E-03	1.95
	EXTRACELLULAR MATRIX	7.60E-03	1.95
	LYSOSOME	3.40E-02	1.38
	EXTRACELLULAR REGION PART	3.50E-02	1.95
	MICROSOSME	1.70E-02	1.06
	INTERMEDIATE FILAMENT	3.70E-02	0.57
	RIBOSOME	5.00E-03	1.84

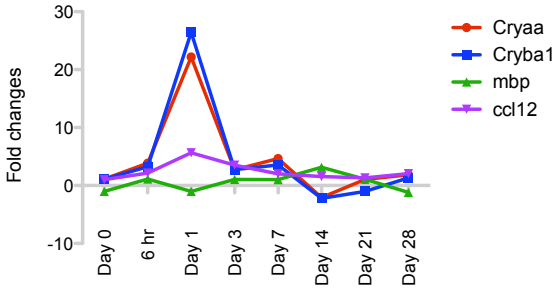
LEGEND KEY

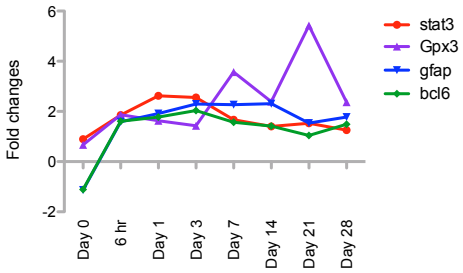
0 DAY UPREGULATED
3 DAY UPREGULATED
7 DAY UPREGULATED
14 DAY UPREGULATED
21 DAY UPREGULATED
28 DAY UPREGULATED

Examples of categories of genes with increased retinal gene expression 0-28 days post ONC.

Changes in Retinal Gene Expression in Ischemia/Reperfusion Model: We utilized Affymetrix mouse gene arrays to identify molecular pathogenic pathways involved in I/R damage to the retina, optic nerve, and superior colliculus. RNA was isolated from these tissues at 0, 0.25, 1, 3, 7, 14, 21 and 28 days after I/R injury in order to determine temporal changes in gene expression. All RNA samples were tested for overall quality using Agilent bioanalyzer chips. In our initial analysis, retinal RNA samples were labeled and hybridized on Affymetrix chips, and the resulting CEL files were analyzed by Partek software to convert signals from chip hybridization to gene lists. We are clustering genes into 3 different categories: biological process, molecular function, and cellular component using DAVID functional annotation bioinformatics to help interpret the data and to select pathways and prioritize genes for further analysis. The following figure demonstrates the temporal nature of changes in retinal gene expression following I/R injury. For example, there is an immediate but short-lived increase in the expression of many crystalline genes (e.g. Cryaa & Cryba1), which are molecular chaperones attempting to protect protein function. In contrast, there is a prolonged increase in the expression of Stat3 (a pathologic

signaling gene) and Gfap (a marker of astrocyte activation). Additional analyses are identifying and confirming pathogenic pathways that will be targeted for future neuroprotection strategies.





Summary of Research Accomplishments

- Systemic treatment with JNK inhibitor (SP600125) totally protected retinal function in mouse model of retinal ischemia/reperfusion (I/R) injury
- Acute administration of neuroprotective estrogen prior to optic nerve crush did not protect retinal ganglion cells in mouse model of optic nerve crush (ONC)
- Discovered in vitro neuroprotective activity of estrogen analogs ZYC-26, ZYC-3, and G1 in cultured 661W neurons subjected to glutamate-induced cytotoxicity. Although these agents are estrogen analogs, they lack feminizing activity.
- Discovered progressive quantitative changes in retinal function assessed by ERG analysis in mouse models of ONC and retinal I/R
- Gaining new insights into retinal, optic nerve, and superior colliculus molecular pathogenic pathways in mouse ONC and I/R models
- Discovered and developed new technique (black-gold staining) to identify and quantify damage to the visual brain centers (superior colliculus)
- Discovered early loss of neurons in superior colliculus in mouse model of ONC
- Discovered mouse strain that develops elevated IOP after Ad5.MYOC.Y437H transduction, which causes optic nerve axonal injury
- Discovered molecular pathogenic pathways in cultured RGCs that are regulated by the sigma-1 receptor
- Updated our cryostat sectioning of mouse eyes and brains with new tape system allowing higher quality and more informative immunohistologic assessment of molecular pathogenesis
- Discovered that intravitreal injection of AAV.Brn3b vector transduced retinal ganglion cells (RGC). This therapy increased Brn3b expression in RGC soma and axons in the optic nerve head and promoted RGC axon regeneration in rat model of chronic ocular hypertension
- Generating AAV2 vector expressing the molecular chaperone BiP, which will be tested for neuroprotective properties in mouse models of ONC and retinal I/R injury
- Presented/will present 10 abstracts at scientific meetings (ARVO and Society for Neuroscince) and submitted 3 scientific manuscripts supported by the VISION project, which acknowledged DoD support

CONCLUSIONS

Upon completion of year 2, we have set up and developed the infrastructure to conduct the proposed 5 year VISION project, including: (1) hiring and training qualified postdoctoral fellows and research technicians, (2) establishing and characterizing 3 mouse models of damage to visual axis tissues (retina, optic nerve, and visual centers in the brain), (3) developing quantitative assays to assess injury-induced damage to the visual axis, (4) identifying and testing 6 defined therapeutic neuroprotective strategies, and (5) using temporal genomics analyses of injured tissues in these mouse models of damage to the visual axis to identify new molecular pathogenic pathways in order to identify new therapeutic strategies. The 3 injury models (optic nerve crush, retinal ischemia/reperfusion, and chronic IOP elevation) use different insults to induce injury to the retina, optic nerve, and visual centers in the brain. We have shown that optic nerve crush not only damages the retina, but also damages the superior colliculus in the brain, so optic nerve crush is a model of retinal and brain injury. We have shown that inhibition of JNK activation with a small molecule JNK inhibitor totally protected retinal function in the acute and severe model of retinal ischemia/reperfusion injury. Although still early in this 5 year project, our research has lead to 10 abstracts and 3 submitted scientific manuscripts. We are extremely concerned about further productivity of this project because we have only been funded for years 1-2 of our 5 year grant proposal. We have requested a no cost extension for year 3 and will have to cut down our overall plans for testing all of the neuroprotective strategies in all of these mouse models of ocular and brain injury. It is truly a shame that the considerable initial investment in the VISION project will not make maximum use of our abilities to discover important new neuroprotective agents to aid our warfighters.

REPORTABLE OUTCOMES:

Publications/Presentations

Phatak NR, Minton AZ, Mireles CE, Krishnamoorthy R. Overexpression of POU domain transcription factor, Brn3b, cuased neurite outgrowth and axon elongation in cultured transformed 661W cells. 2011 ARVO Annual Meeting, Abstract #2659

Mueller B, Krishnamoorthy R, Daudt D, Ma H-Y, Yorio T. Interaction of sigma-1 receptors with voltage gated calcium channels attenuates calcium response in primary retinal ganglion cells. 2011 ARVO Annual Meeting, Abstract #2657

Yorio T, Daudt D, Mueller B. Effects of sigma-1 receptors on mitochondrial fusion in retinal ganglion cells. 2011 ARVO Annual Meeting, Abstract #4619

Nixon ES, Simpkins JW. Neuroprotective effects of non-feminizing estrogen analogues in retinal neurons. 2011 Society for Neuroscience Abstract No. 895.01.

Mueller B, Ma H-Y, Yorio T. Inhibition of NMDA induced calcium ion influx in retinal ganglion cells through sigma-1 receptor stimulation. 2012 ARVO Annual Meeting Abstract

Putliwala T, McDowell C, Liu Y, Casavant TL, Faga B, Thole D, Wordinger RJ, Braun TA, Clark AF. Temporal changes in retinal gene expression after optic nerve crush in mice. 2012 ARVO Annual Meeting Abstract

Kim B-J, Wordinger RJ, Clark AF. Pathologic progression of retinal ischemia and reperfusion injury in mice associated with defective retinal function. 2012 ARVO Annual Meeting Abstract

Liu Y, McDowell C, Tebow H, Beckwith T, Wordinger RJ, Clark AF. Pattern ERG deficits after optic nerve crush in mice. 2012 ARVO Annual Meeting Abstract

Stankowska DL, Minton AZ, He S, Krishnamoorthy R. Neuroregenerative properties of transcription factor Brn3b in an elevated IOP rat model of glaucoma. 2012 ARVO Annual Meeting Abstract

Mueller B, Daudt D, Yorio T. Neuroprotective effects and enhanced retinal ganglion cell synapse formation through sigma-1 receptor mediated mitochondrial fusion. 2012 ARVO Annual Meeting Abstract

Mueller BH, Daudt DR, Ma H-Y, Stankowska D, Crishnamoorthy R, Dibas A, Clark AF, Yorio T. Sigma-1 receptor attenuates calcium influx through L-type

voltage-gated calcium channels in primary retinal ganglion cells. Submitted for publication.

Daudt DR, Mueller B, Park YH, Wen Y, Yorio T. Methylene blue protects primary retinal ganglion cells from cellular senescence. Submitted for publication.

Nixon ES, Simpkins JW. Neuroprotective effects of non-feminizing estrogen analogues in retinal neurons. Invest Ophthalmol Vis Sci, Submitted for publication.

REFERENCES: None

APPENDICES: None